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Synthesis of 3-*O*-sulfated oligosaccharides to understand the relationship between structures and functions of heparan sulfate

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Abstract

The sulfation at the 3-OH position of glucosamine is an important modification in forming structural domains for heparan sulfate to enable its biological functions. Seven 3-*O*-sulfotransferase isoforms in the human genome are involved in the biosynthesis of 3-*O*-sulfated heparan sulfate. As a rare modification present in heparan sulfate, the availability of 3-*O*-sulfated oligosaccharides is very limited. Here, we report the use of a chemoenzymatic synthetic approach to synthesize six 3-*O*-sulfated oligosaccharides, including three hexasaccharides and three octasaccharides. The synthesis was achieved by rearranging the enzymatic modification sequence to accommodate the substrate specificity of 3-*O*-sulfotransferase 3. We studied the impact of 3-*O*-sulfation on the conformation of the pyranose ring of 2-*O*-sulfated iduronic acid using NMR, and on the correlation between ring conformation and anticoagulant activity. We identified a novel octasaccharide that interacts with antithrombin and displays anti factor Xa activity. Interestingly, the octasaccharide displays a faster clearance rate than fondaparinux, an FDA approved pentasaccharide drug, in a rat model, making this octasaccharide a potential short acting anticoagulant drug candidate that could reduce bleeding risk. Having access to a set of critically important 3-*O*-sulfated oligosaccharides offers the potential to develop new heparan sulfate-based therapeutics.

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Supporting Information

Detailed experimental procedures, HPLC chromatograms, MS spectra as well as NMR spectra of synthesized oligosaccharides and additional data figures. This information is available free of charge on the ACS Publications website at <http://pubs.acs.org>.

Competing Interest

J.L. and Y.X. are founders of Glycan Therapeutics, LLC. The other authors declare no competing interest.

TOC image

Introduction

Heparan sulfate (HS) is a polysaccharide comprising disaccharide repeating units of a glucuronic acid (GlcA) or iduronic acid (IdoA) residue linked to a glucosamine (GlcN) residue, with each capable of being modified by sulfation. HS exhibits important physiological and pathological functions, including regulating embryonic development, inflammatory responses, blood coagulation and viral/bacterial infections^{1–3}. Most notably, heparin, a highly sulfated form of HS, is a widely used anticoagulant drug in clinics for the treatment of patients with thrombotic disorders⁴.

The functional selectivity of HS and heparin is governed by the sulfation types and the location of GlcA and IdoA residues⁵. Sulfation is found at the 2-OH of IdoA (and to a lesser extent, GlcA) and the N-, 3-OH and 6-OH positions of GlcN residues. Moreover, the conformation of the IdoA and IdoA2S residues adopt both chair (1C_4) and skew boat (2S_O) conformations⁶. The conformations of GlcA, GlcA2S and GlcN residues exist in the chair (4C_1) conformation^{7–8}. The conformational flexibility of IdoA2S residues is known to be essential for binding to antithrombin to display anticoagulant activity⁹ and fibroblast growth factors to regulate cell growth¹⁰.

The synthesis of HS oligosaccharides remains a challenge. A number of oligosaccharides are synthesized via a purely organic synthetic approach^{11–15}, but it is still very difficult to synthesize oligosaccharides larger than hexasaccharides with complex sulfation patterns. As an alternative approach, a chemoenzymatic method to synthesize HS oligosaccharides has emerged, using HS biosynthetic enzymes involving glycosyltransferases, C₅-epimerase, and sulfotransferases^{16–17}. The method offers high-efficiency synthesis for a wide range of oligosaccharides; however, the synthesis of certain oligosaccharide sequences is not yet possible due to a lack of understanding of the substrate specificities of HS biosynthetic enzymes.

The 3-*O*-sulfation occurs infrequently in HS, but this sulfation type is intimately linked to its biological functions¹⁸. The 3-*O*-sulfation is critically important for anticoagulant activity¹⁹, facilitates the entry of herpes simplex virus into host cells to establish infection²⁰, regulates axon guidance and growth of neurons¹⁸ and controls the progenitor cell expansion for salivary gland development³. Effects in model organisms were also reported, including neurogenesis in *Drosophila*²¹, neurite branching in *C. elegans*²² and cardiac contraction in

zebra fish²³. Precisely how the 3-*O*-sulfated glucosamine (GlcNS3S±6S) residues play critical roles in contributing to the biological activity of HS is currently unknown. The GlcNS3S±6S residue is known to be surrounded by other monosaccharide residues to form a unique sulfated saccharide sequence domain, enabling HS to exert its biological effects. For example, the GlcNS3S±6S residue existing in a pentasaccharide domain enables HS to bind to antithrombin (AT)²⁴. An octasaccharide carrying the GlcNS3S±6S residue interacts with herpes simplex virus glycoprotein D²⁵. Seven isoforms of 3-OST are present in the human genome that may potentially be used to prepare different 3-*O*-sulfated oligosaccharides²⁶.

Results

Chemoenzymatic synthesis of oligosaccharides carrying GlcNS3S and GlcNS3S6S residues

The synthesis of hexasaccharides (compound **1** to **3**, Fig 1A) and octasaccharides (compound **4** to **6**, Fig 1A) was accomplished in the current study. Two 3-*O*-sulfotransferase (3-OST) isoforms, 3-OST-1 and 3-OST-3, were employed to install the GlcNS3S±6S residue into different saccharide sequences. The 3-OST-1 enzyme introduces a sulfation to form a GlcNS3S6S residue that is linked to a GlcA residue at the nonreducing end, forming the disaccharide unit of -GlcA-GlcNS3S6S-; whereas the 3-OST-3 enzyme introduces a sulfation to form a GlcNS3S residue that is linked to an IdoA2S residue at the nonreducing end, forming the disaccharide unit of -IdoA2S-GlcNS3S-. Although 3-OST-1 has been successfully used to synthesize oligosaccharides in numerous studies^{16–17, 27–28}, the use of 3-OST-3 to synthesize oligosaccharides consisting of the -IdoA2S-GlcNS3S- disaccharide units has not been reported.

Here, we discovered that 3-OST-3 and 3-OST-1 have different substrate requirements. The 3-OST-1 enzyme sulfates oligosaccharide substrates that carry 6-*O*-sulfation, while displaying very low reactivity towards the oligosaccharide substrates that lack 6-*O*-sulfation (Supplementary Fig S1), which is consistent with the conclusions described previously¹⁶. In contrast, 3-OST-3 preferentially sulfates oligosaccharides that do not carry 6-*O*-sulfation (Supplementary Fig S1), but the reactivity of those 6-*O*-sulfated oligosaccharide substrates to 3-OST-3 modification was low (Supplementary Fig S1). Results from kinetic analysis demonstrate that 3-OST-3 has higher catalytic efficiency for oligosaccharide substrates without 6-*O*-sulfation as determined by the values of k_{cat}/K_m (Supplementary Fig S2).

The discovery of distinct substrate requirements for 3-OST-1 and 3-OST-3 led us devise two separate schemes to synthesize oligosaccharides that contain different 3-*O*-sulfated saccharide sequences. For the synthesis of oligosaccharides containing the IdoA2S-GlcNS3S±6S disaccharide unit (compound **1**, **2**, **4** and **5**), 3-*O*-sulfation by 3-OST-3 was introduced prior to the 6-*O*-sulfation step (Fig 1B), whereas for the synthesis of oligosaccharides containing the GlcA-GlcNS3S6S disaccharide unit, the 3-*O*-sulfation by 3-OST-1 was performed after the 6-*O*-sulfation step (Fig 1B) (compound **3** and **6**). The AT-binding domain consists of a pentasaccharide unit of -GlcNS(or Ac)6S-GlcA-GlcNS3S±6S-IdoA2S-GlcNS6S-, of which the 3-*O*-sulfation on the central glucosamine residue of this pentasaccharide domain is critical for high binding affinity^{24, 29} (Supplementary Fig S3).

Among all the oligosaccharides tested in this study, only compounds **8*** and **11*** contain the pentasaccharide unit (Supplementary Fig S3).

Structural and conformational analysis of oligosaccharides

The purity and structural analysis of compounds **1** to **6** was conducted; representative data for the analysis of compound **6** is shown in Fig 2. Compound **6** was eluted as a single peak from high resolution anion exchange HPLC, suggesting that the compound is pure (Fig 2B). The molecular mass of compound **6** was determined to be 2449.43 ± 0.74 by electrospray ionization mass spectrometry (ESI-MS), which is very close to the calculated molecular mass of 2448.92 (Fig 2C). The ^1H -NMR spectrum of compound **6** clearly shows eight anomeric protons, confirming that the product is an octasaccharide (Supplementary Fig S24). The ^{13}C -NMR and full NMR assignment for compound **6** are shown in Supplementary Fig S25 and Supplementary Table S1 respectively. The additional structural characterization including full NMR assignments for compound **1** to **5** are shown in Supplementary Figs S4 to S26 and Supplementary Table S1. To locate the 3-*O*-sulfo group in compound **4**, tandem MS analysis was carried out. In this analysis, a stable isotopically labeled [^{34}S]sulfo group was introduced by the 3-OST-3 enzyme, allowing us to unambiguously identify the presence of the 3-*O*-sulfo group at residue d in compound **4** (Supplementary Fig S27). Given the fact that 3-OST-3 prefers sulfating the GlcNS residue that resides internally (residue d) over the one that is close to the reducing end (residue b) of the octasaccharide substrate, the enzyme possibly preferentially modifies the GlcNS residues in the middle of HS polysaccharide chains during the biosynthesis. As compound **4** was the intermediate for compound **5** and **6**, the tandem MS analysis of compound **4** also helped to locate the IdoA2S-GlcNS3S6S disaccharide unit in compound **5** and **6**.

The pyranose rings of IdoA2S residues interconvert between different conformations, including chair forms (1C_4 and 4C_1) and the skew boat form (2S_O) (Fig 1C). Previously, we demonstrated that 2-*O*-sulfation, 6-*O*-sulfation and 3-*O*-sulfation (made by 3-OST-1 enzyme) pushes the equilibrium for IdoA2S residues towards the 2S_O conformer, while these sulfations drive the equilibrium for IdoA residues towards the 1C_4 conformer³⁰. In the present study, the impact of 3-*O*-sulfation on the conformation of neighboring IdoA2S residues was investigated. The conformational analysis was completed by NMR through measuring the three bond proton proton coupling constants ($^3J_{H-H}$)³⁰. The 3-*O*-sulfation increases the population of 2S_O conformer of the IdoA2S residue in hexasaccharides (compound **1** to **3**, Table 1 and Fig 1A), compared to that of the IdoA2S residue in a hexasaccharide without the GlcNS3S6S residue (compound **7***). In compound **3**, the IdoA2S residue almost exclusively displays the 2S_O conformation. For octasaccharides (compound **4** to **6**, Table 1 and Fig 1A), two IdoA2S residues exist, designated as residue c and e. The effect of 3-*O*-sulfation on the 2S_O population varies. For example, 3-*O*-sulfation increases the 2S_O population for both flanked IdoA2S residues as displayed in compound **5**, compared with those of IdoA2S residues in an octasaccharide without the GlcNS3S6S residue (compound **10***). In compound **11***, the 3-*O*-sulfation on residue f increases 2S_O population of the adjacent IdoA2S residue (residue e), but has no effect on a distant IdoA2S residue (residue c), compared with corresponding residues in compound **10***. In compound **6**, 3-*O*-sulfation on residue d and f increases the 2S_O population of residue e, but decreases 2S_O

population of residue c to 58% from 71% (Table 1), compared with the corresponding residues from compound **5**. Our observations raise the possibility that 3-*O*-sulfation could regulate the structure of HS through its effect on the conformation of IdoA2S residues. It has been reported that the IdoA2S residue adjusts its conformation as HS interacts with proteins because of the structural flexibility³¹.

The presence of the -GlcNS3S6S-IdoA2S- disaccharide unit is required for anticoagulant activity

HS oligosaccharides achieve their anticoagulant activity by interacting with AT, therefore oligosaccharides displaying anti-FXa activity should bind to AT. The effects of saccharide residues surrounding the GlcNS3S6S residue on AT-binding as well as anti-FXa activity were examined. Our data revealed that the presence of a -GlcNS3S6S-IdoA2S- disaccharide unit, *i.e.* an IdoA2S located at the reducing end of the GlcNS3S6S residue, is critical in determining anticoagulant activity (Table 2). Compounds **2** and **8*** have six residues, eight sulfo groups, one IdoA2S and two GlcA residues, but only compound **8*** displayed anti-FXa activity (Fig 3A and Table 2). As expected, AT binds to compound **8*** tightly with a K_d value of 7 ± 2 nM, but not to compound **2** (Table 2). Structurally, the compounds differ in the location of the IdoA2S residue. In compound **8***, the 3-*O*-sulfated glucosamine (GlcNS3S6S) residue is flanked by an IdoA2S residue at the reducing end such that it possesses the -GlcNS3S6S-IdoA2S- disaccharide unit, whereas the GlcNS3S6S in compound **2** is flanked by a GlcA residue at the reducing end such that it possesses the -GlcNS3S6S-GlcA- disaccharide unit.

Our findings are consistent with the previously published conclusion that the 2S_O conformation is essential for anticoagulant activity⁹. The IdoA2S residue in compound **8*** displays the 2S_O conformation; whereas in compound **2**, this position is occupied by a GlcA residue that adopts the 4C_1 conformation. The X-ray crystal structure and NMR solution structure of the complex of AT and fondaparinux demonstrate that the IdoA2S residue is present in 2S_O conformation in the complex^{32–33}. We reported that an IdoA-containing 6-mer, where the IdoA2S residue was substituted with an IdoA residue displaying 2S_O conformation, exhibited anti-FXa activity³⁰ (Table 2). However, a GlcA2S-containing 6-mer, where the IdoA2S residue was substituted with a GlcA2S residue displaying 4C_1 conformation, did not display anti-FXa activity⁸ (Table 2). Compound **3** displayed strong anti-FXa activity as the 6-mer consists of two GlcNS3S6S residues (Fig 3A and Table 2), consistent with previously published results for an octasaccharide containing a similar pentasaccharide domain³³. Residues d and c in compound **3** constitute the -GlcNS3S6S-IdoA2S-disaccharide unit.

The GlcA residue in the AT-binding domain is substitutable by an IdoA2S residue

Our studies uncovered a new AT-binding saccharide sequence. The currently known AT-binding sequence comprises a disaccharide -GlcA-GlcNS3S6S-unit³⁴ (Supplementary Fig S3). The substitution of the -GlcA-GlcNS3S6S- disaccharide unit with the -IdoA2S-GlcNS3S6S- disaccharide unit in HS was perceived to abolish AT-binding affinity^{35–36}. The data from the anti-FXa activity and AT-binding analysis of the 8-mers clearly challenged this assertion. Compound **5**, which contains the -IdoA2S-GlcNS3S6S- (but not -GlcA-

GlcNS3S6S-) disaccharide unit, displays strong anti-FXa activity (Table 2). The AT-binding affinity analysis using isothermal titration calorimetry (ITC) also demonstrated that compound **5** binds to AT tightly (Fig 3B and Table 2). It is unsurprising to see compound **6** displaying anti-FXa activity and high AT-binding affinity (Table 2) as this 8-mer contains the –GlcA-GlcNS3S6S- disaccharide unit.

The anticoagulant activity of compound **5** was further confirmed in an *in vivo* experiment using a rat model. To this end, compound **5** was administered and its anti-FXa effect was compared to that of fondaparinux and compound **11***, an anticoagulant octasaccharide reported previously¹⁷. The results demonstrated that compound **5** has comparable anti-FXa potency to that of fondaparinux and compound **11*** (Fig 3C) within 30 min after the drug was administered. However, the anti-FXa effect from compound **5** diminished after 4 hours, while the anti-FXa effect from fondaparinux and compound **11*** persisted after 8 hours (Fig 3C). The drug concentrations in the blood sample were also obtained to determine the clearance rate of each compound *in vivo* (Fig 3D). In comparison with fondaparinux and compound **11***, compound **5** was cleared faster from the animal in the first 2 hours.

The structural promiscuity for residue d in the AT-binding site (Fig 4A) is supported by molecular dynamics (MD) simulations of AT in complex with variously modified HS pentasaccharides. The computational technique was first validated by substituting moieties that are known to be essential for AT-binding within an existing co-crystal structure of AT and fondaparinux^{24, 37}. Calculations for the free energy of binding confirmed that removal of the 3-*O*-sulfate on residue c or substitution of residue b with GlcA destabilized the complex, indicated by significantly weakened interaction energy by 29% and 17%, respectively, which was qualitatively consistent with experimental data. In contrast, substitution of GlcA (residue d) with an IdoA2S in ¹C₄ conformation had no effect on free energy (Fig 4B). Interestingly, substitution with an IdoA2S residue in ²S_O conformation modestly enhanced the binding energy by 12% (Fig 4B and Supplementary Fig S27), suggesting the possibility of a more stable AT/pentasaccharide complex if residue d is IdoA2S in ²S_O conformation. IdoA2S in both conformations were capable of maintaining a similar position of the carboxyl moiety with respect to the protein as was found for the GlcA residue in the simulation of fondaparinux (Fig 4C). Collectively, the MD data support the conclusion that substitution of residue d with an IdoA2S residue does not diminish the binding affinity to AT (Fig 4C), and explain how Compound **5** acts as an active anticoagulant despite lacking the canonical pentasaccharide sequence for AT.

Discussion

Here, we describe schemes using the chemoenzymatic approach to prepare a 3-*O*-sulfated oligosaccharide library. We demonstrate that 3-OST-3 modification, to synthesize oligosaccharides comprising the -IdoA2S-GlcNS3S- or -IdoA2S-GlcNS3S6S- disaccharide unit, must precede the 6-*O*-sulfation step, whereas 3-OST-1 modification can occur only after 6-*O*-sulfation in order to generate the –GlcA-GlcNS3S6S- disaccharide unit. Our finding is supported by the ternary cocrystal structures of 3-OST-1/heptasaccharide/PAP³⁸ and 3-OST-3/tetrasaccharide/PAP³⁹. There were no interactions between 3-OST-3 and the 6-*O*-sulfo groups from the tetrasaccharide substrate, consistent with our conclusion that

oligosaccharide substrates for 3-OST-3 do not require 6-*O*-sulfation. In contrast, interactions between 3-OST-1 and the 6-*O*-sulfo groups from the heptasaccharide substrate were observed, suggesting that 6-*O*-sulfation is required to bind to 3-OST-1. The distinct and unique substrate requirements between 3-OST-1 and 3-OST-3 raise a possibility that 3-*O*-sulfated HS modified by different isoforms of 3-OST are biosynthesized through different pathways.

It was widely accepted that the 3-OST-1 enzyme is responsible for synthesizing anticoagulant HS, whereas the 3-OST-3 enzyme is not^{35–36}. The AT-binding sequences isolated so far all consist of the –GlcA-GlcNS3S6S- disaccharide repeating unit, which is a product of 3-OST-1 enzyme modification^{34, 40}. Although Compound **5**, which is a product of 3-OST-3 enzyme modification, does not contain the –GlcA-GlcNS3S6S- disaccharide unit, it binds to AT and displays anticoagulant activity. Our findings suggest that 3-OST-3 is capable of synthesizing anticoagulant HS, as long as the HS comprises the structural domain similar to that of compound **5**. Interestingly, HS isolated from the liver of *3ost1*–/– null mice displayed anticoagulant activity, suggesting that an alternative pathway for the biosynthesis of anticoagulant HS exists⁴¹. The authors suggested that 3-OST-3 contributed to the synthesis of anticoagulant HS in those mice.

The fast clearance of compound **5** offers a potential new short-acting anticoagulant drug candidate with reduced bleeding risk. A short-acting anticoagulant drug, which can be cleared from the circulation quickly before major bleeding effects develop, would be particularly beneficial to those patients with high bleeding risk⁴². Although unfractionated heparin is an anticoagulant with a short half-life, the concern is that the drug causes heparin induced thrombocytopenia (HIT), a life-threatening side effect⁴³. It has been found that short oligosaccharides smaller than 12-mers⁴⁴ do not bind to platelet factor 4, and thus display no risk of causing HIT. As an octasaccharide, compound **5** is hence expected to have very low risk of causing HIT.

The availability of 3-*O*-sulfated oligosaccharides also opens the opportunity to investigate the casual relationship between saccharide sulfation/conformation and biological functions, a major step forward in dissecting the structure and function relationship of HS. HS 3-OST exists in seven different isoforms. In the present study, we demonstrate that different chemoenzymatic schemes are required for 3-OST-1 and 3-OST-3 to prepare different 3-*O*-sulfated oligosaccharide sequences. The conclusion from this study will guide others to develop the chemoenzymatic method using different 3-OST isoforms, allowing the preparation of more complex HS saccharides with more extensive sulfation patterns involving 3-*O*-sulfated glucosamine residues. These studies will enrich the HS oligosaccharide library to assist HS-related research.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig 1. Structures and synthetic schemes for different HS oligosaccharides

Panel A shows the structures of 11 oligosaccharides used in this study. The synthesis of Compound 1 to 6 was accomplished for the first time. Subsequently, these compounds were subjected to purity and structural analysis as described in the text. The synthesis of Compound 7* to 11* was reported in previous publications^{17, 45}. Panel B shows the schemes for the syntheses of compound 1 to 6. Both starting materials, NS2S 6-mer substrate and NS2S 8-mer substrate, were synthesized in a previous publication¹⁷. 6-OSTs represent 6-*O*-sulfotransferase isoform 1 and isoform 3. Panel C depicts the structures of three conformers of an IdoA2S residue.

Fig 2. Purity and structural analysis compound 6

Panel A shows the structure of the compound 6. Panel B shows the chromatogram of DEAE-HPLC analysis. Panel C shows the ESI-MS spectrum of compound 6. The molecular ions carrying 5, 6, and 7 negative charges are indicated.

Fig 3. Biochemical and biological evaluation of synthesized oligosaccharides

Panel A shows the *in vitro* inhibition curve of the activity of FXa. The data is represented as the mean $\pm$ S.D. (n = 3). Panel B shows the representative data for the ITC analysis of compound **5**. Panel C shows the anti-FXa effects of compound **5**, compound **11*** and fondaparinux in a rat model. The Xa activity measurements at different time points are presented as mean $\pm$ S.D. (n = 4). Compared with the control group (treated with saline), statistical analysis was conducted on each experimental group using a two-tailed Student's t-test, where * represents a p-value of < 0.05. Panel D shows the blood concentrations of compound **5**, **11*** and fondaparinux at different time points in the animals after injection. The concentrations of compound **5**, compound **11*** and fondaparinux were obtained based on the anti-Xa activity, which was converted into the concentration of drugs by standard curves as shown in Supplementary Fig S29. The data are presented as mean $\pm$ S.D. (n = 2).

Fig 4. Molecular Dynamic (MD) analysis of the interaction between AT and fondaparinux
 Panel A shows the structure of AT-binding pentasaccharide. The critical sulfo groups for AT-binding affinity and the IdoA2S residue (residue b) are highlighted in blue. The 2S_O conformation at residue b position is required for binding to AT. The structural requirement for residue d is promiscuous. Panel B depicts the interaction energy ($\Delta\Delta G$) of the five MD simulations of AT complexed with fondaparinux and its derivatives. The graphical representation is expressed as mean $\pm$ SD (* $p < 0.0001$ compared to fondaparinux). Panel C presents an image from three simulations of AT in which residue d of fondaparinux is either GlcA (purple), IdoA2S- $1C_4$ (orange), or IdoA2S- $2S_O$ (green). The simulations were aligned on AT (grey), and the carboxyl groups of residue d are depicted at multiple time points during the simulations (10 frames each from the latter 150 ns).

Table 1

Effects of the GlcNS3S6S or GlcNS3S residue on the population of conformers for IdoA2S in oligosaccharides

Compounds: Abbreviated oligosaccharide structures		Site of IdoA2S residue	Measured $^3J_{H,H}$ couplings (Hz) (Calculated $^3J_{H,H}$ couplings)				Population of conformers ^b			Sum of square difference ^c
			$^3J_{H1-H2}$	$^3J_{H2-H3}$	$^3J_{H3-H4}$	$^3J_{H4-H5}$	1C_4	2S_0	4C_1	
Hexasaccharides	Comp 7* : GlcNS-GlcA-GlcNS-IdoA2S-GlcNS-GlcA-pNP ^d	c	2.2 (2.7) ^a	4.3 (4.1)	3.2 (3.0)	2.3 (2.2)	68 %	32 %	—	0.34
	Comp 1 : GlcNS-GlcA-GlcNS-IdoA2S-GlcNS 3S -GlcA-pNP ^e	c	2.6 (3.1)	5.0 (4.7)	3.3 (3.3)	2.5 (2.3)	58 %	42 %	—	0.38
	Comp 2 : GlcNS6S-GlcA-GlcNS6S-IdoA2S-GlcNS 3S6S -GlcA-pNP	c	4.1 (4.8)	7.8 (7.3)	3.9 (4.3)	3.2 (2.9)	19 %	81 %	—	0.99
	Comp 3 : GlcNS6S-GlcA-GlcNS 3S6S -IdoA2S-GlcNS 3S6S -GlcA-pNP	c	4.8(5.5)	9.0 (8.4)	4.3 (4.8)	3.5 (3.1)	— (1 %)	99 %	—	1.26
	Comp 9* : GlcNAc-GlcA-GlcNS-IdoA2S-GlcNS-IdoA2S-GlcNS-GlcA-pNP	c e	2.5 (3.0) 2.1 (2.5)	4.7 (4.6) 4.0 (3.7)	3.5 (3.3) 3.3 (3.0)	2.5 (2.3) 2.3 (2.1)	62 % 72 %	37 % 24 %	— (2 %) — (3 %)	0.34 0.38
Octasaccharides	Comp 4 : GlcNS-GlcA-GlcNS-IdoA2S-GlcNS 3S -IdoA2S-GlcNS-GlcA-pNP	c e	2.8(3.3) 2.2(2.7)	5.3 (5.1) 4.4 (4.2)	3.5 (3.4) 3.3 (3.1)	2.7 (2.4) 2.5 (2.2)	53 % 67 %	47 % 33 %	— —	0.39 0.42
	Comp 10* : GlcNS6S-GlcA-GlcNS6S-IdoA2S-GlcNS6S-IdoA2S-GlcNS6S-GlcA-pNP	c e	3.1 (3.8) 3.2 (3.8)	6.1 (5.8) 6.1 (5.8)	3.8(3.7) 3.8(3.7)	2.8 (2.5) 2.9 (2.5)	42 % 41 %	58 % 59 %	— —	0.68 0.62
	Comp 5 : GlcNS6S-GlcA-GlcNS6S-IdoA2S-GlcNS 3S6S -IdoA2S-GlcNS6S-GlcA-pNP	c e	3.6 (4.3) 3.9 (4.5)	7.0(6.6) 7.3 (6.9)	4.0(4.1) 4.0(4.2)	3.1 (2.7) 3.0 (2.8)	29 % 25 %	71 % 75 %	— —	0.82 0.60
	Comp 6 : GlcNS6S-GlcA-GlcNS 3S6S -IdoA2S-GlcNS 3S6S -IdoA2S-GlcNS6S-GlcA-pNP	c e	3.0 (3.8) 4.1 (4.8)	6.1 (5.8) 7.8 (7.3)	3.9 (3.7) 3.9 (4.3)	2.9 (2.5) 3.2 (2.9)	42 % 19 %	58 % 81 %	— —	0.93 0.99
	Comp 11* : GlcNAc6S-GlcA-GlcNS 3S6S -IdoA2S-GlcNS6S-IdoA2S-GlcNS6S-GlcA-pNP	c e	3.1 (3.7) 3.6 (4.4)	6.1 (5.7) 7.2 (6.7)	3.7 (3.7) 4.1 (4.1)	2.8 (2.5) 3.1 (2.7)	43 % 27 %	57 % 73 %	— —	0.61 1.05

^aThe calculated values (presented in parentheses) were obtained using Amber 14 with GLYCAM06 parameters ⁴⁶.

^bThe conversion of the measured values of coupling constant to population of different conformers was described in a previous publication ³⁰.

^cThe residual sum of squares (RSS) was used to determine how well the calculated population ratios fit the experimental data.

^dThe population of conformation of comp **7*** was reported in a paper published previously ³⁰.

^eThe position of 3-*O*-sulfated glucosamine (GlcNS3S or GlcNS3S6S) is bolded and underlined.

Table 2

Correlation between the disaccharide domain (–GlcNS3S6S-IdoA2S–) and AT-binding constant (K_d) as well as anti-FXa activity (IC_{50})

	Abbreviated structures	Binding affinity (K_d) to AT (nM)	Anti-FXa activity (IC_{50} , nM)
Fondaparinux, 5-mer	GlcNS6S-GlcA- <u>GlcNS3S6S-IdoA2S</u> -GlcNS6S-OMe ^a	14.8 ± 1.4 nM	12.2 nM
6 – mers (hexasaccharides)			
Comp 8* ^b	GlcNS6S-GlcA- <u>GlcNS3S6S-IdoA2S</u> -GlcNS6S-GlcA-pNP	7 ± 2 nM ^b	9.1 nM
Comp 2	GlcNS6S-GlcA-GlcNS6S-IdoA2S-GlcNS3S6S-GlcA-pNP	No binding	No inhibition
Comp 3	GlcNS6S-GlcA- <u>GlcNS3S6S-IdoA2S</u> -GlcNS3S6S-GlcA-pNP	Not measured	11.0 nM
IdoA-containing 6-mer	GlcNS6S-GlcA- <u>GlcNS3S6S-IdoA</u> -GlcNS6S-GlcA-pNP ^c	32.6 ± 5.3 nM ^d	29.2 nM ^d
GlcA2S-containing 6-mer	GlcNS6S-GlcA-GlcNS3S6S-GlcA2S-GlcNS6S-GlcA-pNP ⁴	No binding ^d	Not measured
8 – mers (octasaccharides)			
Comp 4	GlcNS-GlcA-GlcNS-IdoA2S- <u>GlcNS3S-IdoA2S</u> -GlcNS-GlcA-pNP	No binding ^c	No inhibition
Comp 5	GlcNS6S-GlcA-GlcNS6S-IdoA2S-GlcNS6S-GlcA-pNP- <u>GlcNS3S6S-IdoA2S</u>	5.1 ± 1.4 nM	7.7 nM
Comp 6	GlcNS6S-GlcA- <u>GlcNS3S6S-IdoA2S</u> -GlcNS3S6S-IdoA2S-GlcNS6S-GlcA-pNP	5.6 ± 2.6 nM	10.9 nM
Comp 11 *	GlcNAc6S-GlcA- <u>GlcNS3S6S-IdoA2S</u> -GlcNS6S-IdoA2S-GlcNS6S-GlcA-pNP	8 ± 3 nM ¹	9.4 nM

^aFondaparinux, an FDA-approved anticoagulant, was used as a positive control for anti-FXa activity measurement and the binding to AT. The -GlcNS3S6S-IdoA2S- disaccharide units are presented in bold face and underlined.

^bThe binding constants for comp **8*** and comp **11*** were determined by affinity coelectrophoresis as reported in a previous publication ¹⁷.

^cComp **4** did not bind to AT nor display anti-FXa activity, due to the fact that it does not contain 6-*O*-sulfo groups at the GlcNS residues at the nonreducing end ²⁹.

^dThe IdoA-containing 6-mer was reported in a previous publication ³⁰. IdoA also displays both ¹*C*₄ and ²*S*_O conformations. The GlcA2S-containing 6-mer was reported in a previous publication ⁸.